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(71) Applicant *(for all designated States except US)*: UNIVERSITY OF BATH [GB/GB]; Claverton Down, Bath BA2 7AY (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: POTTER, Barry, V., L. [GB/GB]; 95 Dovers Park, Bathford, Bath BA1 7UE (GB). GUSE, Andreas, H. [DE/DE]; Appener Weg 7a, D–20251 Hamburg (DE). SCHULZE–KOOPS, Hendrik [DE/DE]; Schobertweg 3, D–91056 Erlangen (DE). BERG, Ingeborg [DE/DE]; Nachtigallenweg 11, D–22926 Ahrensburg (DE). MAYR, Georg, W. [DE/DE]; Gaertnerstrasse 4c, D–25421 Pinneberg (DE).

(74) Agents: HARDING, Charles et al.; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).

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(54) Title: CYCLIC ADENOSINE DIPHOSPHATE RIBOSE ANALOGUES FOR MODULATING T CELL ACTIVITY

(57) Abstract

Compounds capable of antagonising a sustained cADPR–mediated rise in intracellular Ca^{2+} levels in a T cell, said rise being in response to stimulation of the T cell receptor/CD3 complex of the T cell, methods for identifying the same and their use in modulating T cell activity are described. The preferred compounds are cyclic adenosine diphosphate ribose analogues.

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THERAPEUTICS

CYCLIC ADENOSINE DIPHOSPHATE RIBOSE ANALOGUES FOR MODULATING T CELL ACTIVITY

The present invention relates to therapeutics. In particular, the present invention relates to the modulation of T cell responses using compounds capable of regulating activation of T cells *via* a cyclic ADP ribose mediated pathway. The invention also relates to treating autoimmune disease and graft rejection using such compounds and methods for identifying such compounds.

Background to the Invention

Adaptive or specific immune responses are normally stimulated when an individual is exposed to a foreign antigen. Specific immunity is mediated by lymphocytes, e.g. B and T lymphocytes. During an immune response, recognition of an antigen leads to activation of lymphocytes that specifically recognise that particular antigen. The lymphocytes proliferate and differentiate into specialised effector cells. The immune response culminates in the development of mechanisms that ultimately eliminate the antigen.

Adaptive immune responses are critical components of host defence during protection against foreign antigens, such as infectious organisms or toxins. However, specific immune responses are also sometimes elicited by antigens not associated with infectious agents, and this may cause serious disease. For example, one of the most remarkable properties of specific immunity is the ability to distinguish between self antigens and foreign antigens. Thus, the lymphocytes in each individual are able to recognise and respond to numerous foreign antigens but are normally unresponsive to potentially antigenic substances present in the individual itself. Unresponsiveness to self antigens is an acquired process that has to be learned by the individual's lymphocytes and has to be maintained throughout life.

Abnormalities in the induction or maintenance of self-tolerance lead to immune responses against self antigens, and debilitating diseases that are commonly called autoimmune

diseases. The spectrum of autoimmune disorders ranges from organ specific diseases (such as thyroiditis, insulitis, multiple sclerosis, iridocyclitis, uveitis, orchitis, hepatitis, Addison's disease, myasthenia gravis) to systemic illnesses such as rheumatoid arthritis or lupus erythematosus.

Another example in which specific immunity against antigens that are not associated with infections causes severe medical problems are rejections of transplanted allografts. In fact, adaptive immune responses to grafted tissues are the major impediment to successful transplantation in most cases.

It is not known what causes the breakdown of tolerance and the initiation of an autoimmune response. However, the mechanisms of tissue destruction in autoimmune diseases and in allograft rejection are essentially the same as those operating in protective immunity. It is generally believed that both autoimmune reactions and allograft rejections are initiated and perpetuated by a response involving T cells. Thus, in the absence of a specific therapy for any of the autoimmune diseases or for allograft rejection, many therapeutic strategies currently employed aim at down modulating the activity of the immune system, in particular by reducing or preventing the activation of T cells.

Recently, monoclonal antibodies to T cell surface antigens, that inhibit T cell activation, or substances that interfere with intracellular T cell activation pathways, such as Cyclosporin A or FK506, have been introduced for the treatment of both allograft rejection and several autoimmune diseases. However, current approaches for the treatment of undesirable T cell activation have been associated with a number of side effects related to general immunosuppression and therefore cannot be considered to be optimal therapy.

Stimulation of T-lymphocytes *via* the T cell receptor/CD3 complex (TCR/CD3) is a critical step in T cell activation and subsequent clonal expansion. Previous studies have shown that activation of the TCR/CD3-complex involves the elevation of the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by at least two mechanisms, a rapid elevation caused by Ca^{2+} release from intracellular stores mediated by inositol (1.4,5) trisphosphate ($Ins(1,4,5)P_3$), and a prolonged elevation that is completely dependent on the influx of extracellular

calcium (reviewed in Guse, 1998). Ins(1,4,5)P_3 appears to play an essential role in Ca^{2+} signalling stimulated *via* the TCR/CD3-complex, since expression of an anti-sense construct directed against the type 1 Ins(1,4,5)P_3 receptor in Jurkat T cells resulted in complete abolition of CD3-mediated Ca^{2+} signalling (Jayaraman *et al*, 1995).

The exact mechanism of Ca^{2+} entry in T cells is still unknown. However, (i) the electrophysiological properties of the Ca^{2+} channels involved, (ii) the absence of Ca^{2+} entry induced by either TCR/CD3 stimulation or Ca^{2+} store depletion in T cells from an immunodeficient patient, and (iii) the observation that mutants in thapsigargin-stimulated Ca^{2+} entry also were defective in TCR/CD3-mediated Ca^{2+} entry argue for the capacitative model in the sustained process of Ca^{2+} entry. Although the exact mechanism of Ca^{2+} entry is still unclear, it is of fundamental importance for a functional immune response since T cells absolutely require the sustained Ca^{2+} signal for clonal expansion. Furthermore, an improved understanding of the signalling pathways involved in T cell activation may be of assistance in developing strategies to suppress inappropriate T cell activity.

Summary of the Invention

We have now shown that the potent Ca^{2+} mobilising compound cyclic ADP-ribose (cADPR), which is found in a variety of eukaryotic cells, is essentially required for sustained Ca^{2+} signalling mediated *via* stimulation of the T cell receptor/CD3 (TCR/CD3) complex. Thus we have shown for the first time that cADPR has a role as a second messenger in T cell activation *via* the TCR/CD3 complex.

Using HPLC analysis (da Silva *et al*, 1998), we have demonstrated that stimulation of the TCR/CD3 complex resulted in activation of a soluble ADP-ribosyl cyclase and a sustained elevation of the intracellular level of cADPR. A causal relationship between elevated cADPR, sustained Ca^{2+} signalling and activation of T cells was revealed by inhibition of TCR/CD3-stimulated Ca^{2+} signalling, cell proliferation and expression of early and late activation markers, CD25 and HLA-DR, using the membrane-permeant antagonist 7-deaza-8-Br-cADPR (WO-A-98/43992). Expression of the molecular target for cADPR in T cells, the type 3 ryanodine receptor/Ca^{2+} channel, was demonstrated both by RT-PCR

and immunoprecipitation/western blotting. Increased cADPR significantly and specifically stimulated the apparent association of [^{3}H] ryanodine indicating a direct modulatory effect on channel opening. In summary, we have shown the presence, causal relation and biological significance of the major constituents of the cADPR/Ca^{2+} signalling pathway in human T cells.

These findings may have important implications for the design of compounds capable of modulating T cell activity since regulation of this cADPR/Ca^{2+} signalling pathway may provide an important means of controlling T cell responses in a variety of T cell mediated immune disorders.

Further, we have also shown that a cADPR antagonist, 7-deaza-8-Br-cADPR, is capable of reducing immune responses in a murine antigen-induced arthritis model. Thus we have demonstrated that a compound that has an effect on the cADPR/Ca^{2+} signalling pathway can inhibit immune responses *in vivo*.

Accordingly the present invention provides a compound for use in modulating T cell activity which compound is capable of antagonising a sustained cADPR-mediated rise in intracellular Ca^{2+} levels in a T cell, said rise being in response to stimulation of the T cell receptor/CD3 complex of the T cell.

Compounds of the invention may be used in treating (i) an autoimmune disease such as an autoimmune disease selected from thyroiditis, insulitis, multiple sclerosis, iridocyclitis, uveitis, orchitis, hepatitis, Addison's disease, myasthenia gravis, rheumatoid arthritis and lupus erythematosus or (ii) allograft rejection.

The elucidation of a novel cADPR-mediated T cell activation pathway will also enable the identification of substances that modulate T cell activation *via* this pathway. Such substances may be used to modulate T cell activity, for example to suppress inappropriate T cell activity in autoimmune reactions or in response to tissue transplantation as an alternative to existing general immunosuppressants such as Cyclosporin A and FK506.

Accordingly, the present invention provides a method for identifying a substance capable of modulating a sustained rise in Ca^{2+} entry *via* a cADPR-mediated pathway which method comprises:

(i) contacting a T cell, which has been stimulated *via* its T cell receptor, with a candidate substance under conditions that would permit a sustained rise in intracellular Ca^{2+} levels in the absence of the substance; and

(ii) determining whether the substance inhibits a sustained rise in intracellular Ca^{2+} levels.

The present invention further provides a method for identifying a substance capable of modulating a sustained rise in Ca^{2+} entry *via* a cADPR-mediated pathway which method comprises:

(i) contacting an ADP-ribosyl cyclase or a homologue, variant or derivative thereof, with a substance to be tested under conditions that would permit the synthesis of cADPR in the absence of the substance; and

(ii) determining whether the substance affects cADPR synthesis.

In one embodiment, the substance inhibits cADPR synthesis, for example reduces or abolishes cADPR synthesis. In another embodiment, the substance modulates, for example inhibits, binding of endogenous cADPR to its receptor binding site.

A compound identified by the methods of the invention may be used in modulating the immune response of a mammal. Thus, for example, in another aspect of the present invention, a compound identified by a method of the invention is provided for use in treating (i) an autoimmune disease, such as thyroiditis, insulitis, multiple sclerosis, iridocyclitis, uveitis, orchitis, hepatitis, Addison's disease, myasthenia gravis, rhematoid arthritis and lupus erythematosus or (ii) allograft rejection.

The present invention also provides a pharmaceutical composition (which term also includes a veterinary formulation) comprising a compound of the present invention, or a pharmaceutically acceptable salt thereof, or a pharmaceutically acceptable solvate of either entity, together with a pharmaceutically acceptable diluent, excipient or carrier.

The invention further provides a compound of the present invention, or a pharmaceutically acceptable salt thereof, or a pharmaceutically acceptable solvate of either entity, or a pharmaceutical composition containing any of the foregoing, for use as a human or animal medicament.

Detailed Description of the Invention

A. Compounds capable of inhibiting cADPR mediated sustained Ca^{2+} signalling in T cells.

Compounds suitable for use in the present invention are capable of inhibiting cADPR-mediated sustained Ca^{2+} signalling in T cells. Preferably, inhibition is specific for the second phase sustained influx of Ca^{2+} resulting from stimulation of the T cell receptor/CD3 complex. In particular, it is preferred that a compound for use in the present invention does not substantially inhibit other pathways involved in the release of Ca^{2+} from intracellular stores, for example $Ins(1,4,5)P_3$ mediated release of Ca^{2+} from intracellular stores. Alternatively, although a compound suitable for use in the present invention may affect to some degree other pathways involved in the release of Ca^{2+} from intracellular stores, it is preferred that the compounds preferentially inhibit cADPR-mediated sustained Ca^{2+} entry into T cells. For example, a preferred compound will inhibit the cADPR pathway at least two-fold, preferably at least 5 or 10-fold more than other pathways involved in Ca^{2+} signalling resulting from TCR/CD3 stimulation.

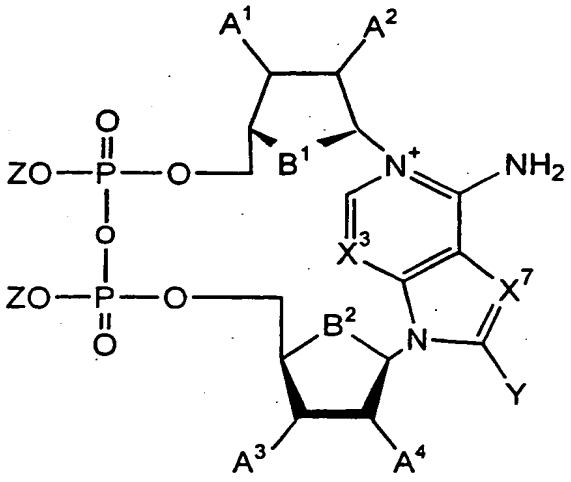
The two phases of Ca^{2+} signalling in T cells have been characterised previously and thus it is well within the capabilities of the skilled person to determine using routine methods whether a compound has an affect on the first phase of Ca^{2+} release or the second, sustained phase of Ca^{2+} signalling. An example of such a method is ratiometric Ca^{2+} imaging.

A compound for use in the present invention may act at a number of places in the cADPR Ca^{2+} signalling pathway. It may affect signalling between the activated TCR/CD3 complex

and ADP-ribosyl cyclase. It may affect activation of a cADPR-metabolising enzyme, for example ADP-ribosyl cyclase (such as covalent modification by an upstream effector protein, for example a kinase), or enzymatic activity of the cADPR-metabolising enzyme (such as a competitive inhibitor having a high binding affinity for the active site of the enzyme or a non-competitive inhibitor which binds a distal site resulting in a conformational change). Alternative, it may affect downstream effects of cADPR (such as channel opening of the ryanodine/Ca^{2+}-channel, which is detectable as modulation of ryanodine binding to ryanodine receptors). In particular preferred compounds such as cADPR analogues may act to inhibit binding of cADPR to its binding site on the ryanodine receptor/Ca^{2+} channel. Suitable assays for identifying compounds for use in the present invention are described below in section B.

One particularly preferred class of compounds for use in the present invention are cADPR analogues, for example a compound comprising an adenine component to which is individually linked two ribose moieties or a derivative(s) thereof, which ribose moieties are joined *via* a pyrophosphate bridging group or a more hydrophobic isostere (i.e. an organic linker of similar size but less polar). Preferably, the ribose moieties are joined to the adenine component at the 1 and 9 positions. Preferably the ribose moieties are joined to the pyrophosphate bridging group or isostere thereof at the 5' position of the ribose rings.

Particular compounds include compounds of formula (1):



(1)

wherein:

A^1, A^2, A^3 and A^4 are independently selected from OH, H, F, NH_2, N_3 and O-hydrocarbyl;

B^1 and B^2 are independently selected from O, S, CHF, CF_2, SO, NR and CH_2

X^3 is independently selected from CR^1 and N;

X^7 is independently selected from CR^2 and N;

Y is halo, C_1 to C_{20} hydrocarbyl, $N(R^3)(R^4)$, OR^5, SR^6 nitro and carboxyl;

each of R, R^1, R^2, R^3, R^4, R^5 and R^6 is independently selected from H and C_1 to C_{20} hydrocarbyl; and

Z is independently selected from H and a group that increases the membrane permeability of the compound;

or a bio-isostere; or a pharmaceutically acceptable salt thereof.

A^1, A^2, A^3 and A^4 are preferably OH or O-acyl. B^1 and B^2 are preferably O. Preferably X^7 is CH and X^3 is N. Y is preferably halo, i.e. chloro, fluoro, iodo (either a natural or a radioisotope such as ^{125}I) or bromo, particularly bromo. Alternatively Y may be amino. Preferably each of R, R^1, R^2, R^3, R^4, R^5 and R^6 is independently selected from H and C_1 to C_{10} hydrocarbyl, more preferably H and C_1 to C_5 hydrocarbyl.

The term "hydrocarbyl group" means a group comprising at least C and H and may optionally comprise one or more other suitable substituents. Examples of such substituents may include halo-, alkoxy-, nitro-, an alkyl group, a cyclic group etc. In addition to the possibility of the substituents being a cyclic group, a combination of substituents may form a cyclic group. If the hydrocarbyl group comprises more than one C then those carbons need not necessarily be linked to each other. For example, at least two of the carbons may be linked *via* a suitable element or group (e.g. carbonyl). Thus, the hydrocarbyl group may contain hetero atoms. Suitable hetero atoms will be apparent to those skilled in the art and include, for instance, sulphur, nitrogen and oxygen.

Preferably, the hydrocarbyl group is any one or more of an alkyl group, an alkylene group, an alkenylene group, an alkenyl group, an alkynylene group, an acyl group or an aryl group, including combinations thereof (e.g. an arylalkyl group) - which groups may optionally

contain one or more heteroatoms or groups, and may further comprise substituents on the chain or rings.

In one preferred embodiment of the present invention, the hydrocarbyl group is a hydrocarbon group.

Here the term "hydrocarbon" means any one of an alkyl group, an alkenyl group, an alkynyl group, which groups may be linear, branched or cyclic, or an aryl group, or combinations thereof (e.g. an arylalkyl group). The term hydrocarbon also includes those groups but wherein they have been optionally substituted. If the hydrocarbon is a branched structure having substituent(s) thereon, then the substitution may be on either the hydrocarbon backbone or on the branch; alternatively the substitutions may be on the hydrocarbon backbone and on the branch. Preferably, at least one of R^{1-6} is H or C_1 to C_4 alkyl.

Compounds of the present invention may contain one or more asymmetric carbon atoms and/or one or more non-aromatic carbon-carbon double bonds and may therefore exist in two or more stereoisomeric forms. Thus, the present invention also provides individual stereoisomers of the compounds of the formula (1), as well as mixtures thereof, including compositions comprising the same. Separation or diastereoisomers or *cis* and *trans* isomers may be achieved by conventional techniques, e.g. by fractional crystallisation, chromatography or HPLC of a stereoisomeric mixture of a compound of the formula (I) or a suitable salt or derivative thereof. An individual enantiomer of a compound of the formula (1) may also be prepared from a corresponding optically pure intermediate or by resolution, such as by HPLC of a racemate using a suitable chiral support or by fractional crystallisation of the diastereoisomeric salts formed by reaction of a racemate with a suitable optically active acid or base.

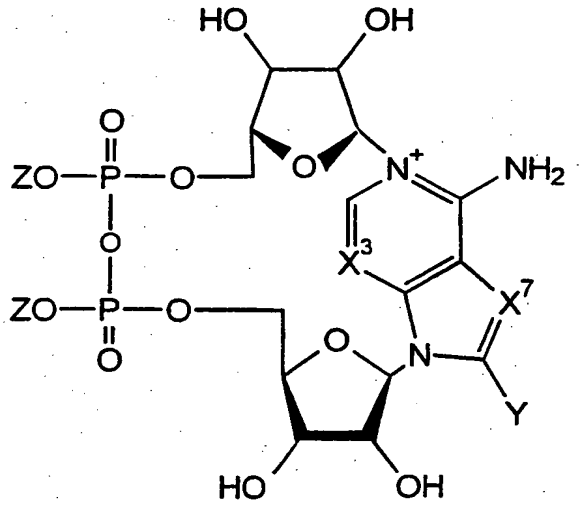
Either both Z groups represent hydrogen atoms (or negative charges) as in a conventional diphosphate system or one Z group may be a group that protects the phosphorous to which it is attached and preferably increases the membrane permeability of the compound (i.e. a hydrophobic group, for example O-acyl groups such as acetoxymethyl), but which

preferably is subsequently removed upon entry into the cell. In a similar manner, one Z group may be a "caging" group e.g. 1-(o-nitrophenyl)ethyl, whereby the compound of the invention is a caged analogue adapted to be converted into an active compound *in situ* e.g. by light irradiation. The chemistry of such caged analogues is well known (see Aarhus *et al.*, 1995; McCray and Trentham, 1989) and such compounds can be prepared by standard methods. The term "caging group" is used in its normal sense - namely a stable group which is labile on irradiation.

The term "bio-isostere" is used in its normal sense - namely a similar (but not the same) or a different chemical structure and having the same biological functional effect.

Preferably the cADPR analogues of formula (1) are substantially non-hydrolysable (the Z group excepted wherein it is other than H).

In a particularly preferred embodiment, a compound for use in the present invention has the formula (2):



(2)

wherein:

X^3 is independently selected from CR^1 and N;

X^7 is independently selected from CR^2 and N;

Y is halo, C_1 to C_{20} hydrocarbyl, $N(R^3)(R^4)$, OR^5, SR^6 nitro and carboxyl;

each of R^1, R^2, R^3, R^4, R^5 and R^6 is independently selected from H and C_1 to C_{20} hydrocarbyl; and

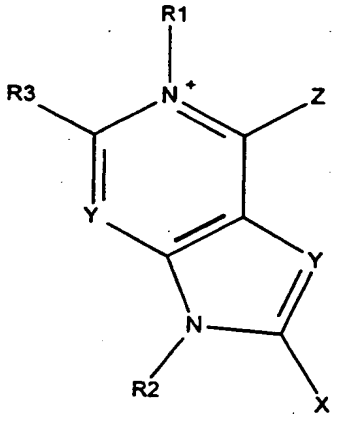
Z is independently selected from H and a group that increases the membrane permeability of the compound;

or a bio-isostere; or a pharmaceutically acceptable salt thereof.

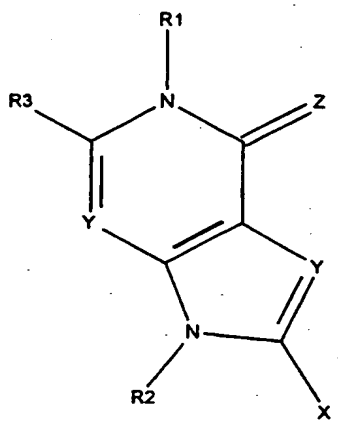
Compounds of formula (2) may be prepared as described in PCT/GB98/00921. Particularly preferred compounds are 7-deaza-8-Br-cADPR and 8-Br-cADPR.

Other preferred compounds include compounds of formula (3) or (4)

Formula (3)



Formula (4)



Where for formula (3), Z is selected from OH, OR, SH, SR^6, NH_2 and NHR^1R^2 and for formula (4), Z is selected from O, S, NH and NHR^1; and

wherein for either formula (3) or formula (4),

Y is selected from N or CH;

X is halo, NH_2 or NHR^1R^2;

R_1 and R_2 are independently selected from H, C_1 to C_{20} hydrocarbyl, sugar moieties and phosphate groups; and

R_3 can be any group but is preferably selected from H and C_1 to C_{20} hydrocarbyl;

or a bio-isostere; or a pharmaceutically acceptable salt thereof.

The terms "bio-isostere", "halo", "R^1", "R^2", "R^6" and "C_1 to C_{20} hydrocarbyl" are defined above.

R_1 and R_2 may be joined *via* a cylic linker group.

Preferably each of R_1, R_2, R_3, R^1, R^2 and R^6 is independently selected from H and C_1 to C_{10} hydrocarbyl, more preferably H and C_1 to C_5 hydrocarbyl.

B. Assays for identifying compounds capable of inhibiting cADPR-mediated sustained Ca^{2+} signalling in T cells.

A substance which affects the cADPR pathway may do so in several ways as discussed above. For example, it may disrupt an interaction between two components of the pathway. In particular, it preferably disrupts the binding of cADPR to its binding site on the ryanodine receptor/Ca^{2+} channel. It may directly disrupt the binding of the two components by, for example, binding to one component and masking or altering the site of interaction with the other component. Candidate substances of this type may conveniently be screened by *in vitro* binding assays. Examples of candidate substances include non-functional homologues of either of the two components as well as antibodies which recognize either of the two components.

A substance which can bind directly to either of the two components may also inhibit an interaction between the two components by altering their subcellular localization thus preventing the two components from coming into contact within the cell. This can be tested *in vivo* using, for example the *in vivo* assays described below. The term '*in vivo*' is intended to encompass experiments with cells in culture as well as experiments with intact multicellular organisms.

Alternatively, instead of preventing the association of the components directly, the substance may suppress or enhance the biologically available amount of one or both of the components. This may be by inhibiting expression of the component, for example at the level of transcription, transcript stability, translation or post-translational stability. An

example of such a substance would be antisense RNA which suppresses the amount of ADP-ribosyl cyclase mRNA translated into protein or antisense RNA which suppresses the amount of type 3 ryanodine receptor mRNA translated into protein.

Suitable candidate substances also include antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies and CDR-grafted antibodies) which are specific for either of the two components. Furthermore, combinatorial libraries, peptide and peptide mimetics, defined chemical entities, oligonucleotides, and natural product libraries may be screened for activity as inhibitors of an interaction between the two components. Other candidate substances include analogues of substrates or products of the cADPR pathway, such as cADPR analogues or NAD analogues.

The candidate substances may be used in an initial screen in batches of, for example 10 substances per reaction, and the substances of those batches which show inhibition tested individually. Candidate substances which show activity in *in vitro* screens such as those described below may then be tested in *in vivo* systems.

In vitro assays typically test for substances capable of affecting the interaction between particular components of the signalling pathway (see above) or the activity of a particular component, such as an enzyme. For example, one assay may involve testing candidate substances for the ability to inhibit synthesis of cADPR by an ADP-ribosyl cyclase. This may be performed by (i) contacting an ADP-ribosyl cyclase with a candidate substance under conditions that would allow the synthesis of cADPR in the absence of the candidate substance and (ii) determining if the candidate substance inhibits cADPR synthesis.

A suitable source of ADP-ribosyl cyclase may be, for example, purified recombinant protein or a crude preparation of T cell extracts. Levels of cADPR may be measured using a combined two-step HPLC method as described in da Silva *et al.* (1998) which has a detection sensitivity for cADPR of about 10 pmol. Typically, cADPR is extracted from cells using perchloric acid. Suitable exogenous substrates for the reaction are NAD^+ or a fluorescent analogue (e.g. 1, N^6-etheno-NAD) and may be added at concentrations of about

100 μM to 1 mM. Alternatively, in the case of cell extracts, endogenous substrates may be sufficient.

Other *in vitro* assays may include assays for identifying substances capable of disrupting ryanodine binding to ryanodine receptors. For example, membrane preparations may be obtained from T cells as described in the Examples and the kinetics of [^{3}H]ryanodine binding to the membrane preparation assessed in the absence or presence of a candidate substance.

A further *in vitro* assay may be used to identify substances that disrupt the binding of cADPR to its receptor on the ryanodine receptor/Ca^{2+} channel. Again, membrane preparations may be obtained from T cells as described in the Examples and the binding of cADPR to the membrane preparation assessed in the absence or presence of a candidate substance. Alternatively, purified or recombinant ryanodine receptor/Ca^{2+} channel may be used instead of membrane preparations (the cloning of a part of the type 3 ryanodine receptor/Ca^{2+} channel is described in the examples).

In vitro assays will generally be used as a preliminary step prior to *in vivo* testing since it is only in the context of an intact TCR/CD3 Ca^{2+} signalling pathway that the modulatory effects of a candidate substance may be completely assessed. *In vivo* assays will typically use either T cell lines, preferably human T cell lines, or T cells obtained from animal tissues, especially human tissues. Generally, T cells will be stimulated *via* the TCR/CD3 complex using standard methods such as receptor cross-linking using antibodies that recognise the receptor complex. They may also be stimulated using superantigens or antigen presenting cells, such as dendritic cells.

A suitable assay method comprises stimulating a T cell *via* its TCR/CD3 receptor in the presence or absence of a candidate substance. The Ca^{2+} response is measured (for example using ratiometric Ca^{2+} imaging) and compared. An effect on sustained Ca^{2+} entry is indicative of a substance capable of modulating T cell activity *via* a cADPR pathway. However, to discount the possibility that the effect may be due to modulation of Ca^{2+} release *via* an Ins(1,4,5)P_3 pathway, it is preferred that the first rapid phase of Ca^{2+} is

substantially unaffected. The sustained phase of Ca^{2+} entry is typically taken to be from approximately 15 minutes after stimulation of the T cell.

T cells may be contacted with a candidate substance before, concomitant with, or after stimulation of the TCR/CD3 complex. The concentration of candidate substance administered to the cell will vary but is typically from 0.1 to 100 μM, more preferably from 1 to 100 μM or 10 to 100 μM.

C. Assays for testing the physiological affects of substances capable of modulating a cADPR pathway.

Since the intention of identifying substances that affect the cADPR pathway is to use them to modulate T cell activity, further assays may comprise administering a substance capable of modulating the activity of the pathway to a T cell, or a candidate substance, and determining the effect on the cell. For example, compounds for use in the present invention are preferably capable of inhibiting or reducing the sustained rise in Ca^{2+} levels following stimulation of the cell *via* the TCR/CD3 complex. Such an affect may include inhibition of cell proliferation in response to, for example, stimulation by an antigen presenting cell such as a dendritic cell. Thus one suitable assay comprises incubating a T cell with an antigen presenting cell in the presence or absence of a candidate substance and determining whether T cell proliferation is reduced in the presence of the substance compared in the absence of the substance.

Another suitable assay involves activating a T cell with a mitogen in the presence and absence of a candidate substance and determining whether T cell proliferation is reduced in the presence of the substance compared in the absence of the substance. Examples of suitable mitogens include monoclonal antibodies to CD3 or the TCR, phorbol 12-myristate 13-acetate, ionomycin, concanavalin A, phytohemagglutinin, superantigens and antibodies to CD2, CD3 or the T cell receptor.

T cell activation/proliferation may be measured using a variety of techniques, for example by measuring levels of secreted cytokines such as IL-2 in the culture medium or by

flowcytometric analysis of T cell surface markers indicative for activation (such as CD69, CD30, CD25 and HLA-DR).

A preferred substance is capable of reducing T cell proliferation by at least 50%, more preferably at least 60, 70, 80 or 90% (for example with respect to numbers of cells expressing a cell surface marker, cytokine levels in the medium and/or numbers of cells present).

Another suitable *in vivo* assay, which is described in example 5, is a murine antigen induced athritis model. Test compounds are typically administered to mice preimmunised with methylated bovine serum albumin (mBSA) in complete Freund's adjuvant, with the experimental arthritis being induced by injection of mBSA into the right knee joint. Compounds are typically administered by intraperitoneal injection.

D. Therapeutic Uses

Compounds capable of affecting a cADPR mediated rise in Ca^{2+} levels in T cells may be used in methods of therapy, for example in treating immune disorders such as autoimmune diseases or graft rejection such as allograft rejection. In particular such compounds may be used to inhibit T cell responses *in vivo*. Alternatively, T cells may be removed from a patient, treated and then returned to the patient (*ex vivo* therapy).

Examples of disorders that may be treated include a group commonly called autoimmune diseases. The spectrum of autoimmune disorders ranges from organ specific diseases (such as thyroiditis, insulitis, multiple sclerosis, iridocyclitis, uveitis, orchitis, hepatitis, Addison's disease, myasthenia gravis) to systemic illnesses such as rheumatoid arthritis or lupus erythematosus. Other disorders include immune hyperreactivity, such as allergic reactions.

In more detail: Organ-specific autoimmune diseases include multiple sclerosis, insulin dependent diabetes mellitus, several forms of anemia (aplastic, hemolytic), autoimmune hepatitis, thyroiditis, insulitis, iridocyclitis, skleritis, uveitis, orchitis, myasthenia gravis,

idiopathic thrombocytopenic purpura, inflammatory bowel diseases (Crohn's disease, ulcerative colitis).

Systemic autoimmune diseases include: rheumatoid arthritis, juvenile arthritis, scleroderma and systemic sclerosis, sjogren's syndrom, undifferentiated connective tissue syndrom, antiphospholipid syndrom, different forms of vasculitis (polyarteritis nodosa, allergic granulomatosis and angiitis, Wegner's granulomatosis, Kawasaki disease, hypersensitivity vasculitis, Henoch-Schoenlein purpura, Behcet's Syndrome, Takayasu arteritis, Giant cell arteritis, Thrombangiitis obliterans), lupus erythematosus, polymyalgia rheumatica, essentiell (mixed) cryoglobulinemia, Psoriasis vulgaris and psoriatic arthritis, diffus fasciitis with or without eosinophilia, polymyositis and other idiopathic inflammatory myopathies, relapsing panniculitis, relapsing polychondritis, lymphomatoid granulomatosis, erythema nodosum, ankylosing spondylitis, Reiter's syndrom, different forms of inflammatory dermatitis.

A more extensive list of disorders is given in WO-A-98/09985. For ease of reference, part of that list is now provided: unwanted immune reactions and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididimo-orchitis, infertility, orchidal trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic

ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fondus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillaim-Barre syndrome, Sydenham chora, myasthenia gravis. pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery or organ, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers, natural or artificial skin tissue.

E. Administration

Compounds capable of affecting a cADPR-mediated rise in Ca^{2+} levels in T cells for use in immunotherapy are typically formulated for administration to patients with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. The formulation will depend upon the nature of the compound identified and the route of administration but typically they can be formulated for topical, parenteral, intramuscular, intravenous, intra-peritoneal, intranasal inhalation, lung inhalation, intradermal or intra-articular administration. The compound may be used in an injectable form. It may therefore be mixed with any vehicle which is pharmaceutically acceptable for an injectable formulation, preferably for a direct injection at the site to be treated, although it may be administered systemically.

The pharmaceutically acceptable carrier or diluent may be, for example, sterile isotonic saline solutions, or other isotonic solutions such as phosphate-buffered saline. The compounds of the present invention may be admixed with any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s). It is also preferred to formulate the compound in an orally active form.

In general, a therapeutically effective daily oral or intravenous dose of the compounds of the invention, including compounds of formula (1) and their salts, is likely to range from 0.01 to 50 mg/kg body weight of the subject to be treated, preferably 0.1 to 20 mg/kg. The compounds of the formula (I) and their salts may also be administered by intravenous infusion, at a dose which is likely to range from 0.001-10 mg/kg/hr.

Tablets or capsules of the compounds may be administered singly or two or more at a time, as appropriate. It is also possible to administer the compounds in sustained release formulations.

Typically, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be

individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

Alternatively, the compounds of the invention, including the compounds of the general formula (1), can be administered by inhalation or in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. An alternative means of transdermal administration is by use of a skin patch. For example, they can be incorporated into a cream consisting of an aqueous emulsion of polyethylene glycols or liquid paraffin. They can also be incorporated, at a concentration of between 1 and 10% by weight, into an ointment consisting of a white wax or white soft paraffin base together with such stabilisers and preservatives as may be required.

For some applications, preferably the compositions are administered orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents.

The compositions (as well as the compounds alone) can also be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. In this case, the compositions will comprise a suitable carrier or diluent.

For parenteral administration, the compositions are best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood.

For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

For oral, parenteral, buccal and sublingual administration to subjects (such as patients), the daily dosage level of the compounds of the present invention and their pharmaceutically acceptable salts and solvates may typically be from 10 to 500 mg (in single or divided doses). Thus, and by way of example, tablets or capsules may contain from 5 to 100 mg of

active compound for administration singly, or two or more at a time, as appropriate. As indicated above, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. It is to be noted that whilst the above-mentioned dosages are exemplary of the average case there can, of course, be individual instances where higher or lower dosage ranges are merited and such dose ranges are within the scope of this invention.

T cells treated *ex vivo* are typically administered to the patient by intramuscular, intraperitoneal or intravenous injection, or by direct injection into the lymph nodes of the patient, preferably by direct injection into the lymph nodes. Typically from 10^4 to 10^8 treated cells, preferably from 10^5 to 10^7 cells, more preferably about 10^6 cells are administered to the patient.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient depending on, for example, the age, weight and condition of the patient.

The present invention will now be described by way of examples which are intended to be illustrative only and non-limiting.

Brief Description of the Figures

Figure 1 - a, b, c - graphs of cADPR levels

Figure 2 - a, b, c, d, e, f - graphs of ratiometric Ca^{2+} measurements

Figure 3 - a, b, c, d - graphs of Ca^{2+} levels

Figure 4 - a, b, c - graphs

Detailed Description of the Figures

Figure 1 - Endogenous cADPR, ADP-ribosyl cyclase activation and $[Ca^{2+}]_i$ in response to stimulation of the TCR/CD3 complex

Jurkat T-lymphocytes (10^8/5 ml intracellular buffer) were left unstimulated for at least 10 min at 37°C. Then cells were stimulated for the times indicated using anti-CD3 OKT3 (10 mg/ml, **a**). Extraction of cADPR by perchloric acid and subsequent neutralization as well as determination of cADPR was carried by a 2-step HPLC procedure using a PRP-X100 and a Hypersil BDS C18 column exactly as described in da Silva *et al.* (1998). Recovery and identity of endogenous cADPR were checked by internal standardisation using authentic cADPR (da Silva *et al.*, 1998). Data for unstimulated cells are from 24 experiments carried out during 1 year; time course analysis for OKT3 was carried out in 5 independent experiments (data presented as mean±SD). In **b,** cells were left unstimulated or exposed to either anti-CD3 OKT3 (10 μg/ml), thapsigargin (1 μM) or ionomycin (200 nM) for 10 min, and endogenous cADPR was determined. Data are presented as mean±SD (n as indicated in the figure). **c**). To determine ADP-ribosyl cyclase activity NAD^+ (100 μM) was incubated with cytosolic (S100) protein (2 mg/ml) at 37°C for 0, 1, 2, 3. 5 and 10 min. The reaction was stopped by perchloric acid (3 M) and cADPR was analysed by reverse phase HPLC as described in da Silva *et al.* (1998). S100 protein was obtained from unstimulated cells (basal activity) or from cells stimulated by OKT3 (10 μg/ml) for 10 min; dephostatin (100 μM) was present during preparation of S100 proteins where indicated. Data are presented as mean±SEM from 4 independent S100 preparations (n as indicated in the Figure). Asterisks denote significant statistical differences to control values according to the Student's t-test ($p > 0.92$). $[Ca^{2+}]_i$ was determined in a suspension of fura-2-loaded Jurkat T cells.

Figure 2 - Inhibition of long-lasting Ca^{2+} signalling by 7-deaza-8-Br-cADPR

$[Ca^{2+}]_i$ was measured in fura-2-loaded Jurkat T cells using a ratiometric single cell Ca^{2+} imaging system (Guse *et al.*, 1997). Cells were either untreated (**a**) or treated with increasing concentrations of 7-deaza-8-Br-cADPR for 20 min (**b** 1 μM, **c** 10 μM, **d** 100 μM) prior to stimulation with anti-CD3 OKT3 (10 μg/ml). In **a** to **d** a typical experiment is displayed (cell number ranging from 20 to 35 cells for each condition). **e** shows $[Ca^{2+}]_i$ at

1500 sec after OKT3 addition as combined data obtained in 5 independent experiments (108 to 203 individual cells investigated for each concentration of 7-deaza-8-Br-cADPR). Asterisks denote significant statistical differences to control values (without 7-deaza-8-Br-cADPR) according to the Student's t-test ($p > 0.97$). **f** shows the effect of 7-deaza-8-Br-cADPR on Ca^{2+} signalling at 1500 sec stimulated by either OKT3 (10 µg/ml), thapsigargin (1 µM) or ionomycin (200 nM); data are presented as mean±SD (3-4 independent experiments, 91 to 104 individual cells investigated; * $p > 0.95$).

Figure 3 - Inhibition of Ca^{2+} signalling by microinjection of antagonists against Ins(1,4,5)P_3 and cADPR

$[Ca^{2+}]_i$ was measured in fura-2-loaded Jurkat T cells using a ratiometric single cell Ca^{2+} imaging system (Guse *et al.*, 1997). Single T cells were stimulated by OKT3 and either left untreated (a: n=10), or microinjected after the first Ca^{2+} spikes were observed with 100 µM 8-methoxy cADPR (b: n=11), 80 µM Ins(1,4,6)P S $_3$ (c: n=10) or a combination of both (d: n=12). Microinjection was carried out exactly as described in Guse *et al.* (1997). Combined data calculated as the relative percentage of the Ca^{2+} peak magnitude before and after microinjection of the respective compound(s) are displayed in (e). Data are presented as mean±SEM. Asterisks denote statistical significances vs. OKT3 data ($p > 0.95$).

Figure 4 - Ryanodine receptor in T-lymphocytes: expression and cADPR effects

Jurkat T cells were disrupted and 2 membrane fractions, P10 (10000 x g pellet) and P100 (100000 x g pellet), and the cytosolic fraction (S100) were prepared by ultracentrifugation.

a, Specific [^{3}H]ryanodine binding to total Jurkat membranes was determined by a filtration assay in the absence or presence of unlabeled ryanodine. Kinetics of the specific binding of 200 nM [^{3}H]ryanodine were investigated in the absence or presence of cADPR (10 µM; n=4-8).

b, The dose-dependency and specificity of the effect of cADPR on the velocity of specific [^{3}H]ryanodine (200 nM) binding was assessed by a 30 min incubation of membranes at 37°C in the presence of different concentrations of cADPR, or for control in the presence of NAD (10 µM) or ATP (10 µM; n=4-8). Asterisks denote significant statistical differences to control values according to the Student's t-test (** $p > 0.90$; * $p > 0.95$).

c, The effect of cADPR (10 μM) on specific binding of 200 nM [^{3}H]ryanodine to P10 membranes (30 min, 37° C) was also measured in the presence of various compounds as indicated (n=3-8). Note that anti-RyR_{common} mAb did not inhibit the effect of cADPR. Asterisks denote significant statistical differences to the value obtained with cADPR alone (second bar) according to the Student's t-test ($p > 0.97$).

EXAMPLES

Materials and Methods

HPLC analysis of cADPR, ratiometric digital Ca^{2+} imaging, microinjection.
These experiments were carried exactly as described by Guse *et al.* (1997) and da Silva *et al.* (1998).

Preparation of subcellular fractions
Jurkat T cells (10^9 cells) were disrupted in 5 ml 20 mM HEPES (pH 7.5), 110 mM NaCl and protease inhibitors (antipain 5 μg/ml, leupeptin 5 μg/ml, pepstatin 6.9 pg/ml, Pefablock SC 13.9 μg/ml) in a Potter-Elvejham homogenizer at 4°C. Then, cell debris was removed at 500 x g for 10 min, and the supernatant was further centrifuged at 10000 x g (20 min, 4°C) and the pellet (P10) harvested. The supernatant was again centrifuged at 100000 x g (120 min, 4°C), and the pellet (P100) and the supernatant (S100) were harvested.

Immunoprecipitation, SDS-PAGE and western blotting
Anti-RyR_{common} mAb was coupled to Protein G-Sepharose and incubated with solubilized P10 membranes in lysis buffer (25 mM HEPES pH 7.2, 150 mM NaCl, 0.25 % CHAPS (w/v), protease inhibitors as above) for 1.5 h at 4°C under continuous shaking. Then, the Protein G-Sepharose beads were spun down (13000 x g, 1 min, 4°C), the supernatant was discarded, and the immunoprecipitate rinsed with lysis buffer; this procedure was then repeated 3 times.

Protein was boiled at 95°C for 5 min and subjected to SDS-PAGE in a 6 % gel (3 % stacking gel) under reducing conditions. Proteins were subsequently transferred onto nitrocellulose sheets by tank blotting (18 h, 550 mA constant, 4°C). The nitrocellulose sheets were washed by a complex procedure and the blots were developed using the ECL-kit (Amersham) according to the manufacturer's instructions.

Binding of [^{3}H]ryanodine to P10 membranes

Binding of [^{3}H]ryanodine was measured in 20 mM HEPES, pH 7.4, 0.75 M KCl, $CaCl_2$ 1.1 mM, EGTA 1.0 mM ($[Ca^{2+}]_{free} \approx 100$ µM) using 200 µg protein in a 50 ml volume at 37°C and continuous shaking. Unspecific binding was determined in the presence of 1000-fold excess of unlabeled ryanodine. Separation of bound from free radioactivity was achieved by rapid filtration on glass fiber filters (Whatman type GF/B). Then, the filters were rinsed rapidly 4 times with 3 ml ice-cold buffer containing 10 mM HEPES, pH 7.4, 0.75 M KCl, $CaCl_2$ 1.1 mM, EGTA 1.0 mM ($[Ca^{2+}]_{free} \approx 100$ µM), and subjected to liquid scintillation counting. Analysis of binding isotherms (at 37°C) resulted in $K_d = 43 \pm 18$ nM (n=3) and a maximal number of specific binding sites of 66±11 fmol/mg protein (n=3).

RT-PCR

To amplify RyR cDNA sequences, PCR was initially performed with cDNA from unstimulated Jurkat T cells and oligonucleotide primers corresponding to a highly conserved sequence from the C-terminal region of all known RyR isotypes. Thus, an antisense primer was designed that was homologous to all 3 human RyR sequences (5' ACATCTTCCAGACATAAG 3') whereas redundant primers were used as sense oligos (5' AC(T/C)CACAATGG(C/G)AAACAG 3'). In addition, specific primers for type 3 RyR were used (sense: 5' CGACATGATGACGTGTTACC 3', antisense: 5' CTCGTACTTGT TCCTGCTGG 3').

Example 1 - Stimulation of T cell Ca^{2+} signalling *via* the TCR/CD3 complex leads to a sustained increase in intracellular cADPR.

Cyclic ADP-ribose (cADPR) has been discovered as a potent Ca^{2+} -mobilizing compound in sea urchin eggs (Lee, 1997 and Galione *et al.*, 1991). In the past decade, it has been

shown that cADPR is also active in plants and in higher eukaryotes including a variety of mammalian tissues or cell types, such as cardiac and smooth muscle, pancreatic and parotid acinar cells, hepatocytes, PC12 cells GH_4C_1 cells, and T-lymphocytes (reviewed in Lee *et al.*, 1997).

We have recently demonstrated in human Jurkat T-lymphocytes (i) that cADPR specifically releases Ca^{2+} from a D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]-insensitive Ca^{2+} pool of permeabilized cells (Guse *et al,* 1995 and Guse *et al,* 1996), (ii) that cADPR stimulates sustained Ca^{2+} signalling in response to microinjection into intact cells (Guse *et al,* 1997), and (iii) that cADPR is an endogenous nucleotide (da Silva *et al.*, 1998).

Despite these findings it remained unclear (i) whether cADPR would act as a second messenger in response to stimulation of the TCR/CD3-complex, and (ii) in which process of Ca^{2+} signalling cADPR would exactly be involved. Previous studies have shown that activation of the TCR/CD3-complex involves the elevation of the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by at least two mechanisms, a rapid elevation caused by Ca^{2+} release from intracellular stores mediated by Ins(1,4,5)P_3 and a prolonged elevation that is completely dependent on the influx of extracellular calcium (reviewed in Guse, 1998). Ins(1,4,5)P_3 appears to play an essential role in Ca^{2+} signalling stimulated *via* the TCR/CD3-complex. since expression of an anti-sense construct directed against the type 1 Ins(1,4,5)P_3-receptor in Jurkat T cells resulted in complete abolishment of CD3-mediated Ca^{2+} signalling (Jayaraman *et al.*, 1995).

The exact mechanism of Ca^{2+} entry in T cells is still unknown. To find out whether cADPR would play a second messenger role in T-lymphocytes a recently developed selective two step HPLC method for quantification of endogenous cADPR was used. The agonistic anti-CD3 antibody OKT3 induced a slowly rising, but sustained increase in intracellular cADPR reaching its highest level 30 min after stimulation, a level that was still maintained after 60 min (Figure 1a). In addition, we have reported that the endogenous inorganic phosphate concentration (P_i) modulates cADPR effects by increased

loading of cADPR-sensitive Ca^{2+} stores (Guse *et al.*, 1995). Since P_i increased in response to OKT3 this may additionally contribute to the action of cADPR.

In contrast to OKT3, stimulation of Ca^{2+} signalling by artificial store depletion using thapsigargin did not result in elevation of endogenous cADPR (Figure 1b). Likewise, elevation of $[Ca^{2+}]_i$ by a moderate concentration of ionomycin which stimulated a Ca^{2+} signal comparable to OKT3, did not increase the level of endogenous cADPR (Figure 1b) indicating that the ADP-ribosyl cyclase involved in TCR/CD3 signalling is not unspecifically stimulated by increased $[Ca^{2+}]_i$. Stimulation of T cells by OKT3 in the absence of extracellular Ca^{2+} resulted in a comparable time course of cADPR as shown in Figure 1a (data not shown).

As a potential link between TCR/CD3-stimulation and increased cADPR concentration, a novel ADP-ribosyl cyclase was detected at low basal activity in the cytosolic fraction of Jurkat cells (Figure 1c). In contrast to known ADP-ribosyl cyclases, like the soluble one from *Aplysia californica* (Hellmich and Strumwasser, 1991) or the plasma membrane bound ectoenzyme CD38 (Howard *et al.*, 1993), the soluble ADP-ribosyl cyclase from Jurkat T cells did not use nicotinamide guanine dinucleotide as substrate (data not shown). Most importantly, the initial reaction velocity of this enzyme was increased significantly (about 2.8-fold) in T cells stimulated by OKT3 (Figure 1c). Moreover, when the cells were stimulated by OKT3 and, subsequently, the cytosolic fraction was prepared in the presence of the Tyr-phosphatase inhibitor dephostatin (Imoto *et al.*, 1993), an about 5-fold increase of cyclase activity was observed (Figure 1c) indicating activation by Tyr-phosphorylation of the soluble cyclase.

Example 2 - The cADPR antagonist 7-deaza-8-Br-cADPR inhibits TCR/CD3-mediated Ca^{2+} entry.

To finally prove a causal relationship between the TCR/CD3-mediated elevation of cADPR and $[Ca^{2+}]_i$, the following approaches were used. First, Jurkat T cells were preincubated with the novel membrane-permeant cADPR-antagonist 7-deaza-8-Br-cADPR and subsequently Ca^{2+} signalling stimulated by OKT3 was measured using digital ratiometric

Ca^{2+} imaging (Figure 2). On the single cell level OKT3 stimulated rapid and sustained Ca^{2+} signalling (Figure 2a). Individual cells responded in different ways: oscillations, longlasting elevations, single spikes; however, the majority of cells showed Ca^{2+} signalling for more than 20 min after stimulation (Figure 2a). Such longlasting responses were never observed in the absence of extracellular Ca^{2+} (data not shown), and therefore mainly reflect Ca^{2+} entry.

Preincubation with 1 μM 7-deaza-8-Br-cADPR did not significantly change the pattern of Ca^{2+} signalling (Figure 2b). However, at higher concentrations (10 and 100 μM) 7-deaza-8-Br-cADPR induced a profound inhibitory effect on the long-lasting Ca^{2+} entry (Figure 2c,d). Statistical analysis revealed a highly significant reduction of Ca^{2+} signalling at 1500 sec after stimulation, suggesting an essential role of cADPR in the important process of Ca^{2+} entry (Figure 2e). In addition 7-deaza-8-Br-cADPR dose-dependently increased the delay between OKT3 addition and the onset of the Ca^{2+} signal (Figure 2c,d) indicating that cADPR is already involved in the early period of Ca^{2+} signalling. An non-specific effect of 7-deaza-8-Br-cADPR on Ins(1,4,5)P_3-induced Ca^{2+} release is rather unlikely because the closely related derivative 8-Br-cADPR has been shown up to 100 μM to be without any inhibitory effect on Ins(1,4,5)P_3-induced Ca^{2+} release in T cells. Importantly, 7-deaza-8-Br-cADPR did not inhibit the sustained Ca^{2+} signalling stimulated by either store-depletion using thapsigargin nor by unspecific elevation of $[Ca^{2+}]_i$ by ionomycin (Figure 2f).

Figure 2c and d also demonstrate that the membrane-permeant antagonist 7-deaza-8-Br-cADPR did not influence the first phase of Ca^{2+} signalling, which is thought to be driven mainly by Ins(1,4,5)P_3. Indeed, the novel membrane-permeant Ins(1,4,5)P_3 antagonist xestospongin C, which has been shown to potently block Ins(1,4,5)P_3-mediated Ca^{2+} release, but up to an extracellular concentration of 20 μM to be without effect on RyR-mediated Ca^{2+} release, inhibited TCR/CD3-mediated Ca^{2+} signalling after preincubation of the cells for 20 min at 2 μM (data not shown). Our results, together with the data from the type 1 Ins(1,4,5)P_3-receptor knock-out T cell line, indicate (i) that an initial Ins(1,4,5)P_3-mediated Ca^{2+} signalling event is necessary, but not sufficient for sustained Ca^{2+} signalling, (ii) that cADPR is essential for the sustained second phase, and (iii) that cADPR is also involved in determining the time of onset of Ca^{2+} signals after TCR/CD3 stimulation.

Example 3 - cADPR antagonists but not IP_3 antagonists inhibit the second phase, sustained rise in Ca^{2+} levels in response to TCR/CD3 stimulation.

The membrane-permeant antagonists 7-deaza-8-Br-cADPR and xestospongin C are powerful tools, but to achieve a sufficiently high intracellular effective concentration, a substantial preincubation period must be employed. To find out whether either Ins(1,4,5)P_3 or cADPR or both play an essential role also at a later time point after TCR/CD3 stimulation, we stimulated the cells by OKT3, and, after development of Ca^{2+} signalling, microinjected a specific antagonist, e.g. inositol 1,4,6-trisphosphorothioate (Ins(1,4,6)PS $_3$), or 8-methoxy-cADPR, or a combination of both (Figure 3). Following the development of Ca^{2+} signalling stimulated by OKT3, microinjection of 8-methoxy-cADPR, a novel cADPR antagonist of similar potency as 8-NH_2-cADPR, significantly decreased the magnitude of further Ca^{2+} signals (Figure 3a, b, e), whereas microinjection of Ins(1,4,6)P S $_3$ did not have a significant effect (Figure 3a, c ,e). Combined microinjection of 8-methoxy-cADPR and Ins(1,4,6)P S $_3$resulted in a somewhat more pronounced inhibition as compared to 8-methoxy-cADPR alone (Figure 3e). These data confirm the concept of both an initial Ca^{2+} signalling phase for which Ins(1,4,5)P_3 is essential, and a sustained phase driven mainly by cADPR.

Example 4 - Functional ryanodine receptors are expressed in Jurkat T cells and their activity modulated by cADPR.

Ca^{2+} release by cADPR has been proposed to proceed *via* ryanodine receptors (RyR) (Galione *et al.*, 1991). Recently, we obtained pharmacological evidence that cADPR acts *via* RyR in T cells. Ca^{2+} release stimulated by cADPR in permeabilised T cells was effectively inhibited by (i) ruthenium red and (ii) high Mg^{2+} concentrations. Both ruthenium red and high Mg^{2+} concentrations are proven as inhibitors of activation of RyR. In contrast, there was no inhibition of Ins(1,4,5)P_3-induced Ca^{2+} release by these treatments indicating a specific antagonistic effect on RyR. Despite these results and the fact that the Ca^{2+} mobilising effects of cADPR in T cells have been reported independently by different groups, the expression of RyR in T cells has been a matter of debate. RyRs have been

detected in T cells by binding studies and western blot and by northern blot analysis. Sequencing of a cDNA clone obtained from Jurkat T cell poly(A)$^+$ RNA showed 95 % identity on the amino acid level to rabbit brain RyR in the C-terminal region. In contrast, transcripts for RyRs have not been detected in another Jurkat clone using RT-PCR. We report here that RyR is clearly expressed in the Jurkat T cell line employed in our laboratory as assessed by western blot and immunoprecipitation experiments using an anti-RyR_{common} mAb. A band of about 500 kDa was detected specifically in P10 membranes prepared from Jurkat cells, but not in the cytosolic fraction (data not shown). Furthermore, immunoprecipitation of solubilised P10 membranes with anti-RyR_{common} mAb resulted in profound enrichment of the RyR (data not shown). These data were confirmed by RT-PCR of poly(A)$^+$ RNA from Jurkat T cells. Using redundant primers for all RyR subtypes, a PCR product of the expected size was obtained; the nucleotide sequence from the amplified product was identical with a sequence from type 3 RyR. A sequence identical to a type 3 RyR sequence was also obtained when specific primers for the type 3 RyR were used (data not shown).

In addition, specific binding of [^{3}H]ryanodine to Jurkat membranes was observed. Importantly, there was a significant stimulatory effect of cADPR on the apparent association velocity of [^{3}H]ryanodine, but not on the capacity of [^{3}H]ryanodine binding to Jurkat membranes (Figure 4a). These findings indicate a direct modulatory effect on channel opening by cADPR, since [^{3}H]ryanodine is suggested to bind the RyR in the open conformation. A similar kinetic effect of cADPR has been reported for Ca^{2+} release from cardiac and rat brain microsomes. The stimulatory effect of cADPR on the binding velocity of ryanodine was dose-dependent (Figure 4b) and specific because (i) it was not observed with ATP or NAD (Figure 4b), and (ii) it was reversed by either high Mg^{2+} concentration, ruthenium red, 8-methoxy cADPR or 7-deaza-8-Br-cADPR (Figure 4c). Importantly, both high Mg^{2+} concentration and ruthenium red decreased [^{3}H]ryanodine binding below control levels whereas the cADPR-antagonists only reversed the stimulatory effect of cADPR (Figure 4c).

Example 5 - Sustained Ca^{2+} signalling *via* a cADPR pathway is necessary for the activation of peripheral T-lymphocytes

Sustained Ca^{2+} signalling is necessary for activation of peripheral T-lymphocytes. The experiments described so far point towards an important role of cADPR in this process. To confirm this, peripheral T cells were activated by solid-phase bound anti-CD3 mAb in the presence of increasing concentrations of 7-deaza-8-Br-cADPR. Proliferation was inhibited partially by 10 mM, and completely by 100 mM 7-deaza-8-Br-cADPR (Table 1). Similarly, the expression of the T cell activation markers CD25 and HLA-DR was markedly suppressed by increasing concentrations of 7-deaza-8-Br-cADPR (Table 1). Most importantly, after a 5 day exposure of the cells to 100 mM 7-deaza-8-Br-cADPR more than 70 % of the cells still were detected in the lymphocyte gate; of these > 95 % were negative for propidium iodine staining, indicating almost no non-specific cytotoxic effect of the antagonist.

Table 1 - Highly purified human peripheral blood T cells (5×10^4) were pulsed with the indicated inhibitors for 30 min at 37°C in the absence of serum, and then transferred to flat bottom 96 well plates coated with anti-CD3 mAb and cultured in RPMI 1640 containing 10 % NHS. The cells were incubated for 96 h in the presence of the indicated inhibitors. [^{3}H]Thymidine was added for the last 16 h of culture. Results are given as mean cpm ±SEM for quadruplicate determinations of one of three independent experiments with similar results (3 different healthy donors). Expression of CD25 and HLA-DR was determined by staining with directly labelled mAbs to CD25 and HLA-DR and analysed by flow cytometry. Histograms were gated for viable lymphocytes. Cyclosporin A (CsA) was added for comparison as an established inhibitor of T cell activation.

Table 1

Addition	[^{3}H]thymidine incorporation [cpm ± SEM]	expression of CD25 (% pos. cells)	expression of HLA-DR (% pos. cells)
Control	148494±17414	24.5	6.0

7-deaza-8-Br-cADPR (1 μM)	156730±13335	16.1	7.6
7-deaza-8-Br-cADPR (10 μM)	94124±9020	14.1	5.3
7-deaza-8-Br-cADPR (100 μM)	42±8	9.8	2.7
CsA(50 ng/ml)	41807±16424	12.7	4.9

Summary

In conclusion, we propose that cADPR is formed as an essential second messenger in response to stimulation of the TCR/CD3-complex and mediates the second, sustained phase of Ca^{2+} signalling *via* ryanodine receptor/Ca^{2+} channels in T cells. Although we have delineated the signalling pathway from the TCR/CD3-complex in the plasma membrane up to the RyR, the exact mechanism by which cADPR regulates Ca^{2+} entry is not yet clear. However, since cADPR by releasing Ca^{2+} from its target store also mediated Ca^{2+} pool depletion, it might act in a similar way as suggested for $Ins(1,4,5)P_3$ in the classical capacitative model.

Finally, our data indicate that modulation of the cADPR signalling pathway in T cells may have important therapeutic implications. Starting from the membrane permeant antagonist 7-deaza-8-Br-cADPR novel pharmaceuticals may be developed that can be used to modulate undesirable immune reactions, e.g. immune hyperreactivity, autoimmune diseases and graft rejection.

Example 6 - Treatment of antigen-induced arthritis (AIA) in mice by antagonists of cyclic ADP- ribose (cADPR)

It has now been shown in the above examples that antagonists of cADPR specifically inhibited activation of human T-lymphocytes as measured by inhibition of intracellular Ca^{2+}-signalling, expression of activation antigens and proliferation of the cells. T-lymphocytes are involved in the genesis of various autoimmune diseases, e.g. rheumatoid

arthritis. An established animal model for rheumatoid arthritis is the antigen-induced arthritis (AIA) in mice. We have therefore examined the effects of a cADPR antagonist, 7-deaza-8-Br-cADPR, on AIA in mice.

Experimental procedures

Female C57BL/6 mice were immunised at day –21 and day –14 with methylated bovine serum albumin (mBSA) in complete Freund's adjuvant. The experimental arthritis was induced at day 0 by injection of mBSA into the right knee joint.

Treatment from day 0 (6h after injection of mBSA) to day 21 was carried out by daily intraperitoneal injection of 100 µl of either vehicle: 0.9 % NaCl solution (n=8), 7-deaza-8-Br-cADPR 0.2 µmol/kg (n=5) and as positive control: Lipotalon (Dexamethasonpalmitat) 500 µg/kg (n=9).

During the treatment period the following parameters were investigated:

swelling of right knee joint: the swelling was determined at days 3, 5, 7, 14 and 21. Swelling was calculated as relative swelling compared to the thickness of the joint at day 0.

body weight: weight was determined at days 3, 5, 7, 14 and 21. Body weight was calculated as relative body weight as compared to the body weight at day 0.

Results

The specific cADPR antagonist 7-deaza-8-Br-cADPR was applied at the very low dose of 0.2 µmol/kg. A reduction in joint swelling was observed at day 3, 5, 7 and 14 as compared to the vehicle alone.

These data, as a proof of principle, indicate that autoimmune diseases can be successfully treated by antagonists of cADPR.

The specific cADPR antagonist 7-deaza-8-Br-cADPR did not significantly change the body weight during the course of treatment. In marked contrast, the anti-inflammatory drug dexamethasone (lipotalon) induced a significant reduction in body weight.

These data suggest that therapy of autoimmune diseases by antagonists of cADPR does not have a general toxic effect on the animals since during the course of therapy (21 days), 7-deaza-8-Br-cADPR did not induce visible toxic effects.

Conclusion: The new data provide the proof of principle for the beneficial effects of antagonists of cADPR in the therapy of autoimmune diseases.

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12. A method according to claim 11 wherein the substance inhibits cADPR synthesis.

13. A method for identifying a substance capable of modulating a sustained rise in Ca^{2+} entry *via* a cADPR-mediated pathway which method comprises:

(i) contacting a T cell, which has been stimulated *via* its T cell receptor, with a candidate substance under conditions that would permit a sustained rise in intracellular Ca^{2+} levels in the absence of the substance; and

(ii) determining whether the substance inhibits a sustained rise in intracellular Ca^{2+} levels.

14. A compound identified by the method of claim 11, 12 or 13 for use in treating or preventing an immune disorder.

15. A compound identified by the method of claim 11, 12 or 13.

16. A process comprising the steps of:

(a) performing the method according to claim 11, 12 or 13;

(b) preparing a quantity of those one or more substances identified as being capable of modulating a sustained rise in Ca^{2+} entry *via* a cADPR-mediated pathway.

17. A process comprising the steps of:

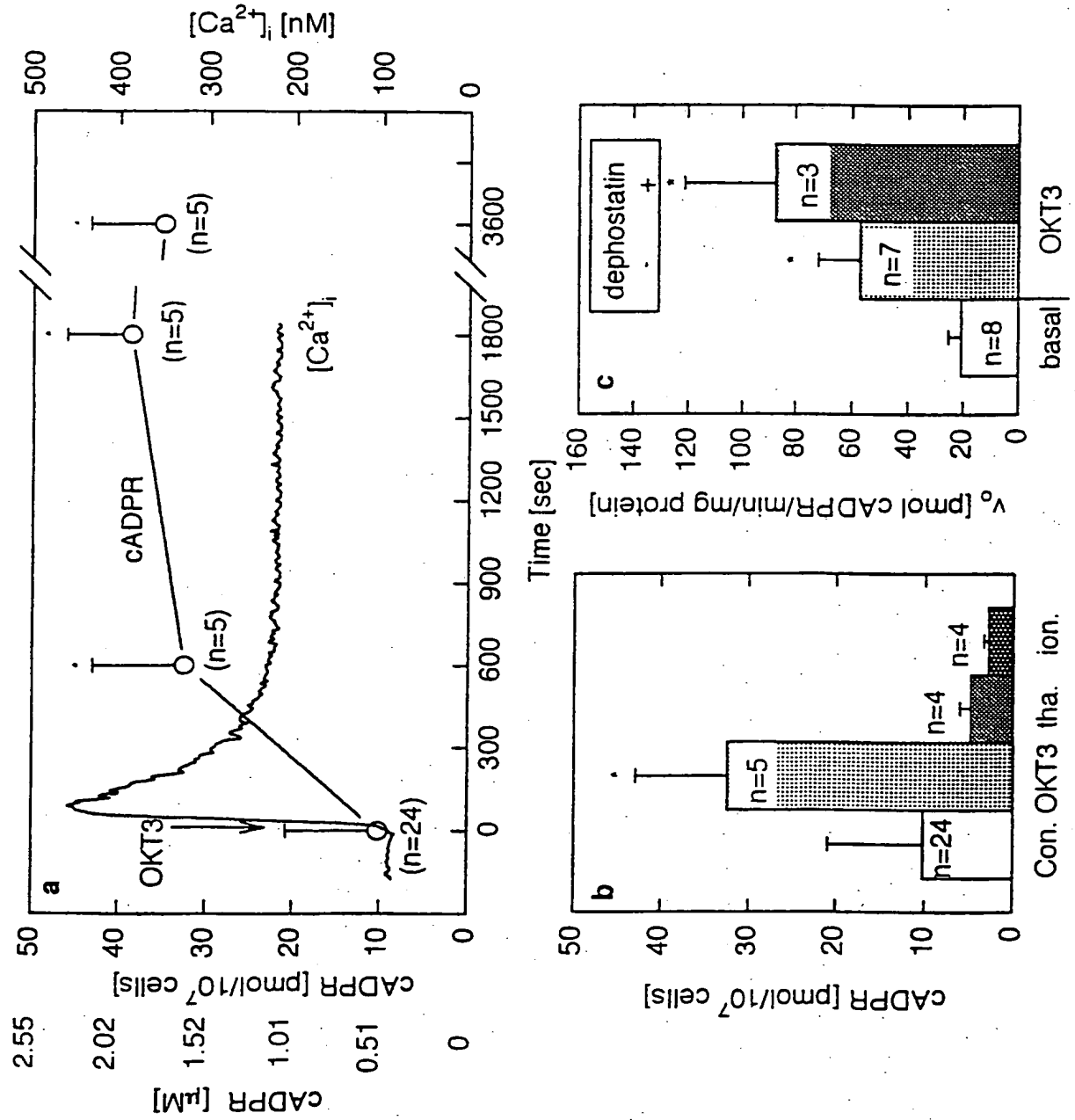
(a) performing the method according to claim 11, 12 or 13; and

(b) preparing pharmaceutical composition comprising one or more substances identified as being capable of modulating a sustained rise in Ca^{2+} entry *via* a cADPR-mediated pathway.

18. A process comprising the steps of:

(a) performing the method according to claim 11, 12 or 13; and

(b) modifying one or more of the substances identified as being capable of modulating a sustained rise in Ca^{2+} entry *via* a cADPR-mediated pathway to cause a different effect on T cell activity.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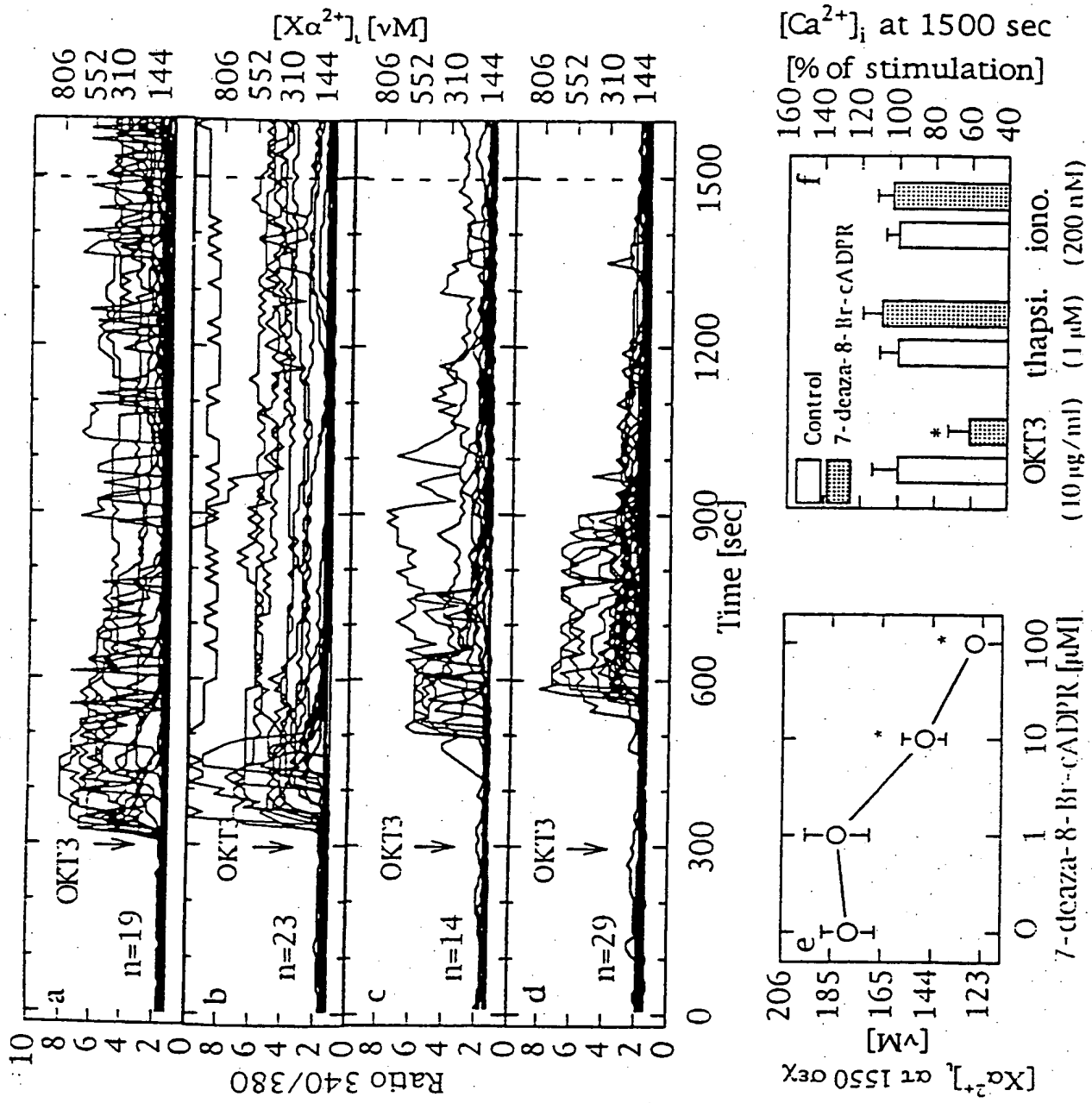
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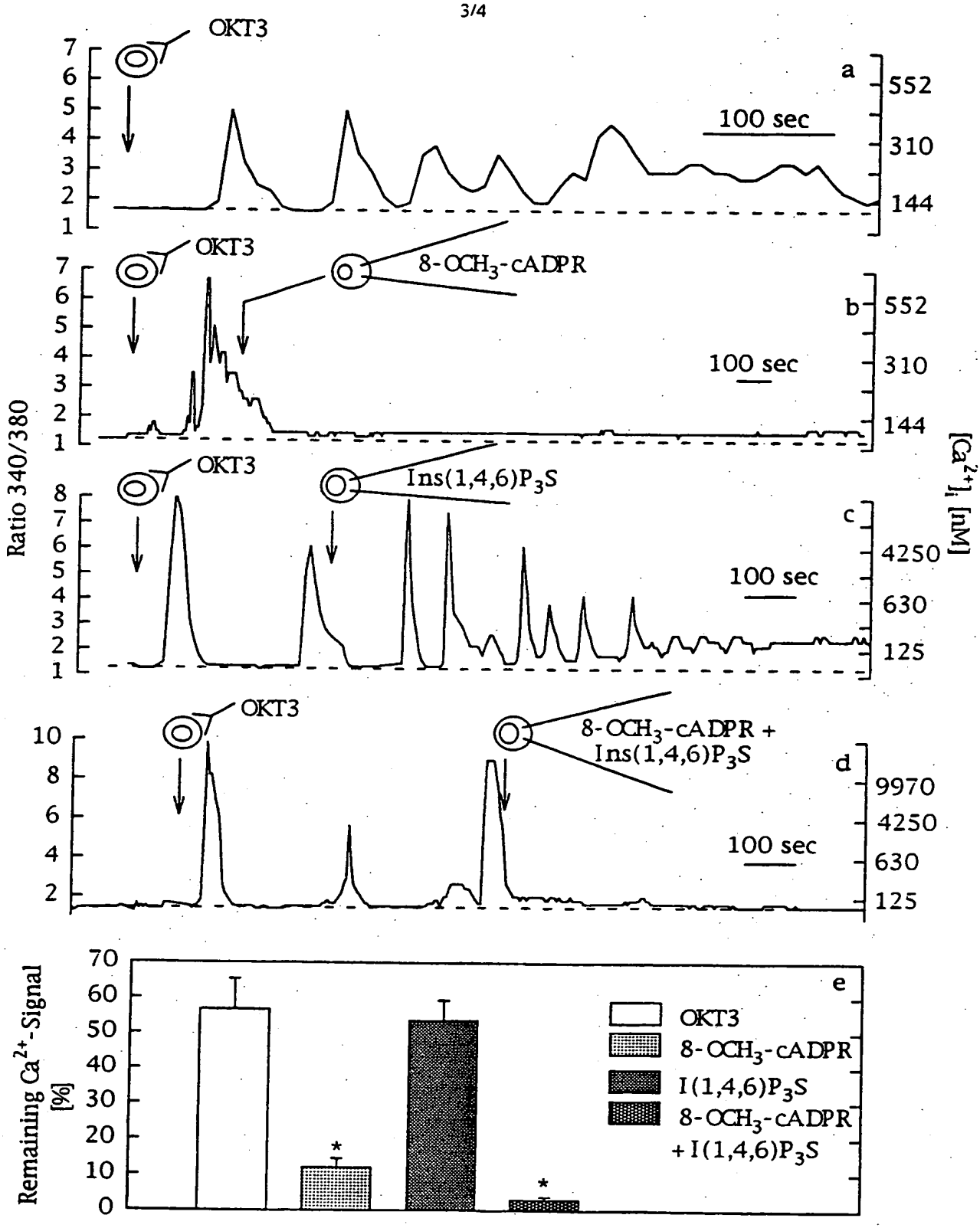
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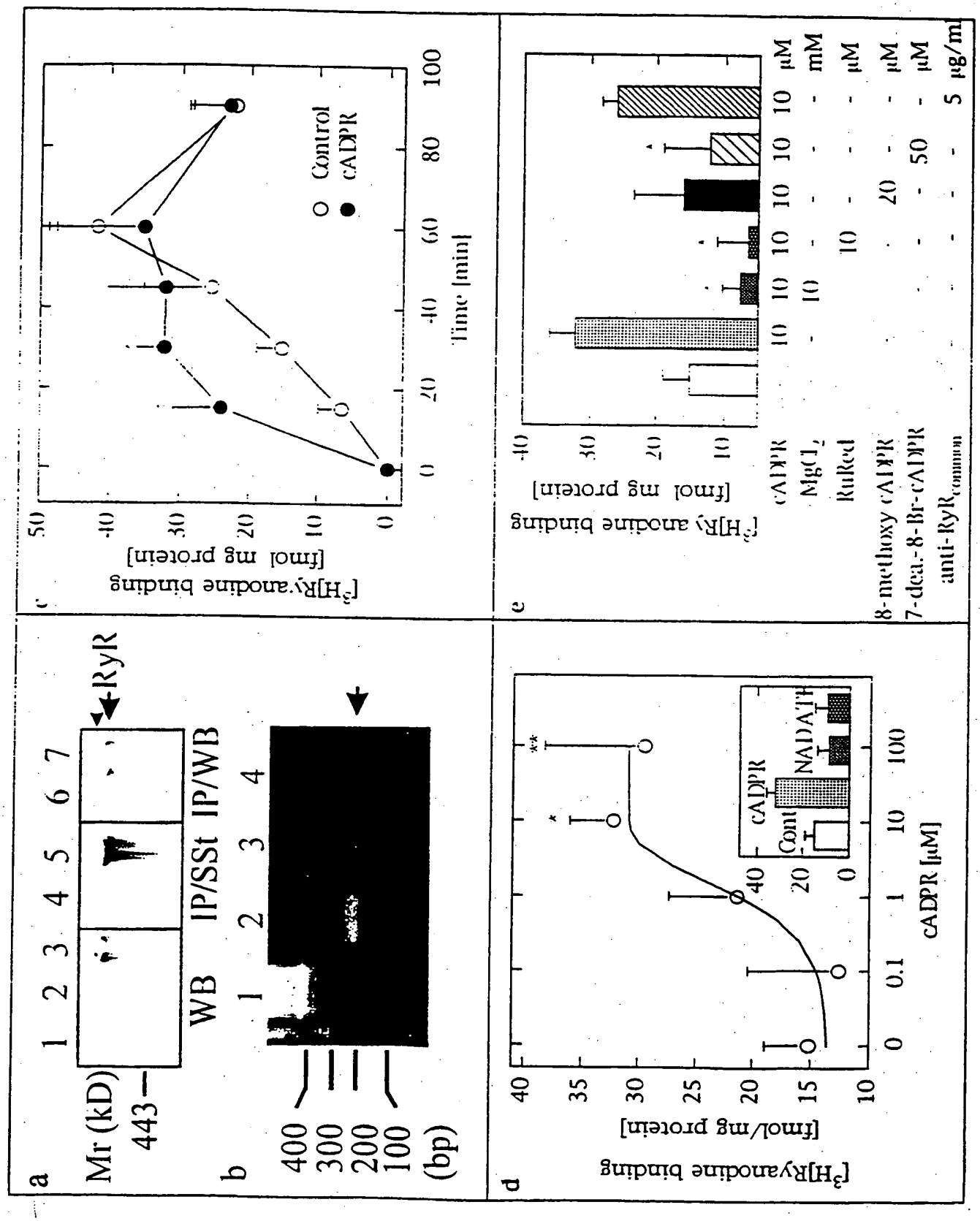
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INTERNATIONAL SEARCH REPORT

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PCT/GB 99/04295

A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 43992 A (UNIV BATH ;GALIONE ANTONY (GB); ISIS INNOVATION (GB); POTTER BARRY) 8 October 1998 (1998-10-08) cited in the application page 12, line 28 -page 13, line 1; claims	11-13, 15-18
Y		1-18
P,X	EP 0 953 572 A (BIOMOLECULAR ENGINEERING RESEA) 3 November 1999 (1999-11-03) claims	1-18

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[X] Further documents are listed in the continuation of box C.

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